TRANSMITTAL LETTER TO THE UNITED STATES **DESIGNATED / ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371**

ATTORNEY'S DOCKET NUMBER P67678US0

US APPLICATION 07.0376FR9527

INTERNATIONAL APPLICATION NO PCT/NZ00/00176 INTERNATIONAL FILING DATE

7 September 2000

PRIORITY DATE CLAIMED 7 September 1999

TITLE OF INVENTION

SEEDLESS FRUIT PRODUCTION

APPLICANT(S) FOR DO/EO/US

Jialong YAO and Bret MORRIS

Applicant herein submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information.
1. This is a FIRST submission of items concerning a filing under 35 U.S.C. 371.
2. This is a SECOND or SUBSEQUENT submission of items concerning a filing under 35 U.S.C. 371.
This express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1).
4. A proper Demand for Internatl. Preliminary Examination was made by the 19th month from earliest claimed priority date.
5. A copy of the International Application as filed (35 U.S.C. 371(c)(2))
a. is transmitted herewith (required only if not transmitted by the International Bureau).
b. as been transmitted by the International Bureau.
c. \square is not required, as the application was filed in the United States Receiving Office (RO/US)
6. A translation of the International Application into English (35 U.S.C. 371(c)(2)).
7. Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))
a. are transmitted herewith (required only if not transmitted by the International Bureau).
b. have been transmitted by the International Bureau.
c. have not been made; however, the time limit for making such amendments has NOT expired.
d. have not been made and will not be made.
8. A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
9. An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).
10. A translation of the annexes to the Internatl. Preliminary Examination report under PCT Article 36 (35 U.S.C. 371(c)(5)).
Items 11. to 16. below concern other document(s) or information included:
11. An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
12. An assignment document for recording. A separate cover sheet compliance with 37 CFR 3.28 and 3.31 is included.
13. A FIRST preliminary amendment.
A SECOND or SUBSEQUENT preliminary amendment.
14. A substitute specification.
15. A change of power of attorney and/or address letter.
16. Other items or information:
International Search Report
PCT Request Form First Page of Publication
Demand
International Preliminary Examination Report - with no annexes
Sequence Listing

10	1/069521	PCT/NZ00	/00176	P6767	8US0						
				CALCULATIONS	PTO USE ONLY						
17. The following fees											
Basic National Fee (37 (CFR 1.492(a)(1)-(5)):										
Internatl. prelim. examina	ition fee paid to USPT0	O (37 CFR 1.492 (a) (1)) \$710.00								
No international prelimina (a) (2)) but international s	ary examination fee pai search fee paid to USP	id to USPTO (37 CFR TO (37 CFR 1.445(a)	1.492 (2)) \$740.00								
Neither international preli nor international search f											
International preliminary ((a) (4)) and all claims sat	examination fee paid to isfied provisions of PC	o USPTO (37 CFR 1.4 T Article 33(2)-(4)	\$100.00								
Search Report prepared	by the EPO or JPO (37	CFR 1.492 (a) (5)) .	\$890.00								
	ENTER APPRO	PRIATE BASIC FE	E AMOUNT =	\$ 1040.00							
Surcharge of \$130.00 for	_			\$							
☐ 20 ☐ 30 months fro		· · · · · · · · · · · · · · · · · · ·									
Claims	Number Filed	Number Extra	Rate								
Total Claims	34 - 20 =	-14-	x \$18.00	\$ 252.00							
Independent Claims	9 - 3 =	-6-	x \$84.00	\$ 504.00							
Multiple Dependent Clain	n(s) (if applicable)		+ \$280.00	\$							
	TOTAL	OF ABOVE CALC	CULATIONS =	\$ 1796.00							
Reduction by 1/2 for filing Entity statement must als	g by small entity , if appose be filed. (Note 37 CF	plicable. Verified Sma R 1.9, 1.27, 1.28).	all	\$							
			SUBTOTAL =	\$ 1796.00							
Processing fee of \$130 fo	-			\$							
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Fee of \$40.00 for recording Assignment must be according to the second s	ng the enclosed assig ompanied by appropria	nment (37 CFR 1.21(te cover sheet (37 CF	h)). R 3.28, 3.31).	\$ 40.00							
		TOTAL FEES	FNCLOSED =	\$ 1836.00							
		TOTALTELO		Amt. to be refunded:	\$						
				Amt. charged:	\$						
a. A check in the amount of \$1836.00 to cover the above fees is enclosed. b. Please charge my Deposit Account No06-1358_ in the amount of \$ to cover the above fees.											
A duplicate copy of t	this sheet is enclosed.										
c. The Commissioner i pendency of this ap this sheet is enclose	s hereby authorized to plication, or credit any ed.	charge my account a overpayment to Depo	ny additional fees esit Account No. 0	set forth in §1.492 (<u>)6-1358</u> . A duplicat	during the e copy of						
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400 7th S Wash 202	SON HOLMAN Platreet, N.W., Suite ington, DC 20004-638-6666	e 600 4	By John Reg	Moly C. Holman No. 22,769	JPH&S 3/95						

10/069527 - JC13 Rec'd PCT/PTO 0 6 MAR 2002

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants: Jialong YAO et al.

Serial No.: New

Filing Date: March 6, 2002

FOR: SEEDLESS FRUIT PRODUCTION

Commissioner of Patents Washington, D.C. 20231

Sir:

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Prior to initial examination, please amend the above-identified application as follows:

IN THE SPECIFICATION

On page 1, immediately following the title, please insert the following sentence: --This is a nationalization of PCT/NZ00/00176 filed September 7, 2000 and published in English.--

IN THE CLAIMS

Please amend claims 3, 8, 11, 12, 19, 20, 25, 26, 29, 33 & 34 as follows:

- 3. (amended) A fruiting plant according to claim 1 which produces a pome fruit.
- 8. (amended) A plant as claimed in claim 4 wherein said plant is one which produces pome fruit.

- 11. (amended) A plant as claimed in claim 8, in which said polynucleotide (b) has the coding sequence of SEQ ID NO: 3.
- 12. (amended) A plant as claimed in claim 8, wherein said polynucleotide (b) has the nucleotide sequence of SEQ ID NO: 3.
- 19. (amended) A DNA construct which includes a polynucleotide as claimed in claim 13.
- 20. (amended) A DNA construct comprising, in the 5'-3' direction:
 - (a) a promoter sequence;
 - (b) an open reading frame polynucleotide as defined in claim 13; and
 - (c) a termination sequence.25. (amended) A DNA construct as claimed in claim 23 [or claim 24] in which the non-coding region is in a sense orientation.
- 25. (amended) A DNA construct as claimed in claim 23 in which the non-coding region is in a sense orientation.
- 26. (amended) A DNA construct as claimed in claim 23 in which the non-coding region is in an anti-sense orientation.
- 29. (amended) A transgenic cell of a fruiting plant which includes a DNA construct as claimed in claim 19.
- 33. (amended) A seedless or sterile fruit which is produced by a fruiting plant as claimed in claim 1.
- 34. (amended) A seedless or sterile pome fruit which is produced by a fruiting plant as claimed in claim 3.

REMARKS

The foregoing Preliminary Amendment is requested in order to delete the multiple dependent claims and avoid paying the multiple dependent claims fee.

Attached hereto is a marked-up version of the changes made to the specification and claims by the current amendment. The attached page is captioned " <u>VERSION</u> <u>WITH MARKINGS TO SHOW CHANGES MADE."</u>

Early action on the merits is respectfully requested.

Respectfully submitted,

JACOBSON HOLMAN PLLC

By

John C. Holman

Reg. No. \22,769

400 Seventh Street, N.W. Washington, D.C. 20004-2201 (202) 638-6666

Atty. Docket: P67678US0 Date: March 6, 2002

JCH/cmf

VERSION WITH MARKINGS TO SHOW CHANGES MADE

- 3. (amended) A fruiting plant according to claim 1 [or claim 2] which produces a pome fruit.
- 8. (amended) A plant as claimed in <u>claim 4</u> [any one of claims 4 to 7] wherein said plant is one which produces pome fruit.
- 11. (amended) A plant as claimed in claim 8, [claim 9 or claim 10] in which said polynucleotide (b) has the coding sequence of SEQ ID NO: 3.
- 12. (amended) A plant as claimed in claim 8, [claim 9 or claim 10] wherein said polynucleotide (b) has the nucleotide sequence of SEQ ID NO: 3.
- 19. (amended) A DNA construct which includes a polynucleotide as claimed in <u>claim 13</u> [any one of claims 13 to 18].
- 20. (amended) A DNA construct comprising, in the 5'-3' direction:
 - (a) a promoter sequence;
 - (b) an open reading frame polynucleotide as defined in <u>claim 13</u> [any one of claims 13 to 18]; and
 - (c) a termination sequence.25. (amended) A DNA construct as claimed in claim 23 [or claim 24] in which the non-coding region is in a sense orientation.

- 25. (amended) A DNA construct as claimed in claim 23 [or claim 24] in which the non-coding region is in a sense orientation.
- 26. (amended) A DNA construct as claimed in claim 23 [or claim 24] in which the non-coding region is in an anti-sense orientation.
- 29. (amended) A transgenic cell of a fruiting plant which includes a DNA construct as claimed in claim 19 [any one of claims 19 to 28].
- 33. (amended) A seedless or sterile fruit which is produced by a fruiting plant as claimed in <u>claim 1</u> [any one of claims 1, 2, 4-7 and 31].
- 34. (amended) A seedless or sterile pome fruit which is produced by a fruiting plant as claimed in claim 3 [any one of claims 3, 8 to 12 and 32].

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of: Jialong YAO et al.

Filed: March 6, 2002

Art Group: To Be Assigned

Serial No.: 10/069,527

Examiner: To Be Assigned

For: SEEDLESS FRUIT PRODUCTION

AMENDMENT AND SUBMISSION OF SUBSTITUTE SEQUENCE LISTING UNDER 37 C.F.R. §1.825(a)

Honorable Commissioner of Patents and Trademarks Washington, D.C. 20231

Dear Sir:

In response to the Notification of Missing Requirements Under 35 U.S.C. 371 Application Papers mailed May 10, 2002, kindly amend the captioned application as follows:

AMENDMENT

In the Sequence Listing:

Please replace the existing Sequence Listing for the above-identified application with the Substitute Sequence Listing appended hereto.

REMARKS

Submission of Substitute Sequence Listing

In connection with the Substitute Sequence Listing submitted herewith, the undersigned hereby states that:

- 1. In accordance with 37 C.F.R. 1.825(a), the Substitute Sequence Listing does not contain new matter.
- 2. In accordance with 37 C.F.R. 1.825(b), the content of the attached paper copy and the attached computer readable copy of the Substitute Sequence Listing are the same.
- 3. All statements made herein are true and that all statements made on information and belief are believed to be true, and that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent resulting therefrom.

Applicants also enclose herewith two floppy disks containing the computer readable form of the Substitute Sequence Listing and a copy of the Notice to File Corrected Application Papers.

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CONCLUSION

It is respectfully believed this application is now in condition for allowance. Early notice to this effect is earnestly solicited.

It is not believed that extensions of time or other are required beyond those that may otherwise be provided for herewith. However, if additional extensions of time are necessary to prevent abandonment of this application, then such extensions of time are hereby petitioned under 37 C.F.R. §1.136(a), and any fees required therefor (including fees for net addition of claims) are hereby authorized to be charged to our Deposit Account No. 06-1358, Attorney Docket No. P67678US0.

Respectfully submitted,

JACOBSON HOLMAN, PLLC

Date: June 26, 2002

The Jenifer Building 400 Seventh Street, N.W. Washington, DC 20004-2201

(202) 638-6666

3

13 hecd PCT/PTO 2 7 JUN 2002 10/069527

SEQUENCE LISTING

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                                185
 Leu Ala Leu Ser Asn Gly Ala Asn Asn Leu Tyr Thr Phe His Leu His
                             200
                                                205
His Pro Asn Leu His His Gly Gly Ser Ser Leu Gly Ser Ser Ile Thr
                         215
His Leu His Asp Leu Arg Leu Ala
                     230
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                                                                    30
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<223> Description of Artificial Sequence: Made in lab
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                                                                   20
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SEEDLESS FRUIT PRODUCTION

FIELD OF THE INVENTION

5 The invention provides plants that produce seedless or sterile fruit.

BACKGROUND TO THE INVENTION

The production of seedless or parthenocarpic fruit is a desirable trait for commercially grown cultivars. Seedless fruit are more convenient than seeded fruit to consumers. Furthermore parthenocarpic fruit trees can be cropped without pollination, which reduces dependence on bees, pollinator varieties and warm weather at flowering. The absence of pollen is also advantageous so as to alleviate environmental concerns regarding the transfer of transgenes to non-transgenics by cross-pollination.

Seedless fruit cultivars can also avoid or reduce biennial bearing tendencies that have been attributed to the inhibition of flower bud formation by developing seeds in apple (Chan and Cain, 1967). Seedless apple fruit is also much less susceptible to codling moth, a major pest on apple trees, compared to seeded fruit (Goonewardene et al., 1934).

The applicants have now identified and isolated a reproductive gene which encodes a peptide involved in the reproductive (seed-producing) cycle of fruiting plants, particularly apple trees. It is broadly towards this gene, to its homologs in other fruiting plants and to the modulation of its expression/function within fruiting plants that the present invention is directed.

SUMMARY OF THE INVENTION

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In a first aspect, the present invention provides a fruiting plant which has been genetically modified such that it does not functionally express:

(i) a peptide having the MdPl amino acid sequence of SEQ ID NO: 2 or a functionally equivalent variant thereof; and/or

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- (ii) a peptide having the *MdAP3* amino acid sequence of SEQ ID NO: 4 or a functionally equivalent variant thereof,
- 5 which plant produces seedless or sterile fruit.

In a further aspect, the invention provides a fruiting plant which contains a polynucleotide encoding a peptide having the *MdPI* amino acid sequence of SEQ ID NO: 2 or a functionally equivalent variant thereof and in which the functional expression of said peptide within said plant has been disrupted such that the plant produces seedless or sterile fruit.

In still a further aspect, the invention provides a fruiting plant which contains:

- 15 (a) a polynucleotide encoding a peptide having the *MdPI* amino acid sequence of SEQ ID NO: 2 or a functionally equivalent variant thereof; and
- (b) a polynucleotide encoding a peptide having the *MdAP3* amino acid sequence of SEQ ID NO: 4 or a functionally equivalent variant thereof,

and in which the functional expression of said peptide encoded by polynucleotide

(a) within said plant has been disrupted such that the plant produces seedless or

sterile fruit.

In one form, functional expression of said peptide encoded by polynucleotide (a) is disrupted directly.

In another form, functional expression of said peptide encoded by polynculeotide (a) is disrupted indirectly, such as through disrupting functional expression of the peptide encoded by said polynucleotide (b).

As used herein, "fruiting plant" means a plant in which the fruit is formed from the ovary and the fused bases of sepals, petals and stamen, whereas "functional

expression" of said peptide refers to the amount of the peptide which is expressed and functional within the plant. For example, a plant which does not functionally express a peptide can mean either that there is no expression of that peptide at all, or that the peptide is expressed but no longer performs its previous function.

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Conveniently, the plant is one which produces a pome fruit.

Disruption of functional expression may be by mutation (such as frameshift, deletion, insertion or knockout mutations) of the gene itself or of its regulatory elements, down-regulation (such as antisense, co-suppression) or any other method known to those skilled in the art by which aberrant or reduced expression of the gene may be achieved (e.g. Montgomery and Fire, 1998).

Disruption may therefore be specifically caused by down-regulation of expression of *MdPI* by down-regulation of expression of inter-related *MdAP3*, or both.

In a further embodiment, the invention provides a polynucleotide which encodes a peptide having the *MdPI* amino acid sequence of SEQ ID NO: 2 or a variant thereof, or which encodes a peptide having the *MdAP3* amino acid sequence of SEQ ID NO: 4 or a variant thereof.

Most preferably, said polynucleotide includes part or all of the nucleotide sequence of SEQ ID NO: 1, or part or all of the nucleotide sequence of SEQ ID NO: 3.

25 Preferably, the polynucleotide is DNA.

The invention further provides a DNA construct which includes a polynucleotide as defined above.

More particularly, the invention provides a DNA construct comprising, in the 5'-3' direction:

(a) a promoter sequence;

- (b) an open reading frame polynucleotide coding for the peptide having the *MdPI* amino acid sequence of SEQ ID NO: 2 or a functionally equivalent variant thereof; and
- (c) a termination sequence.

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In one embodiment, the open reading frame is in a sense orientation.

In an alternative embodiment, the open reading frame is in an anti-sense orientation.

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In still a further embodiment, the invention provides a DNA construct comprising, in the 5'-3' direction:

- (a) a promoter sequence;
- 15 (b) a non-coding region of a gene coding for the peptide having the
 MdPI amino acid sequence of SEQ ID NO: 2 or a functionally
 equivalent variant thereof; and
 - (c) a termination sequence.
- 20 Once again, the non-coding region can be in a sense or anti-sense orientation.

In yet a further embodiment, the invention provides a DNA construct comprising, in the 5'-3' direction:

- 25 (a), a promoter sequence;
 - (b) a polynucleotide comprising a nucleotide sequence complementary to at least part of a sequence coding for the peptide having the *MdPI* amino acid sequence of SEQ ID NO: 2 or a functionally equivalent variant thereof; and
- 30 (c) a termination sequence.

Preferably, in each embodiment, the construct further includes a marker for identification of transformed cells.

Similar constructs can also be provided including a polynucleotide which encodes part or all of the MdAP3 peptide having the sequence of SEQ ID NO: 4.

In still a further aspect, the invention provides a transgenic fruiting plant cell which includes a DNA construct as defined above, as well as a transgenic fruiting plant comprising such cells.

Finally, the invention includes seedless or sterile fruit produced by a plant as defined above.

DESCRIPTION OF THE DRAWINGS

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While the invention is broadly defined as above, those persons skilled in the art will appreciate that it is not limited thereto and that it also includes embodiments of which the following description provides examples or which are the subject of specific claims. In addition, the present invention will be better understood from reference to the accompanying drawings in which:

Figure 1 shows the phenotype of wild type and Rae Ime apple flowers and fruit.

(a) normal apple flowers showing sepals, petals, stamens and styles.

(b) a normal 5-week-old apple fruit showing five carpels with 0 to 2 seeds per carpel.

(c) Rae Ime flowers with no petals or stamens but with increased numbers of styles.

(d) cross sections at the lower part (left) and upper part of a 5-weekold Rae Ime fruit, showing two whorls of carpels without seed.

(e) top of Rae Ime fruit showing two whorls of calyxes.

(f) top of normal apple fruit showing a whorl of calyxes.

(g) ' mature fruit of Rae Ime with size of 5 cm wide and no seed.

Figure 2 shows the sequence of MdPI. The cDNA sequences and deduced amino acid sequences of MdPI isolated from Granny Smith apple are shown. Gene specific PCR primers are underlined. Primer directions are indicated with horizontal arrows. Intron positions are indicated with vertical arrows.

Figure 3 shows a Northern blot analysis of apple RNA sample using *MdPI* cDNA as a probe. RNA sample were prepare from ovaries (1), sepals (2), young leaves (3), skin (4), cortex (5) and core (6) tissue of 4-week-old fruit of Granny Smith, 1-week-old fruit (7), flower peduncles (8), stamens (9), petals (10) of Granny Smith (12), flower buds of Rae Ime (11), and flower buds of Granny Smith (12).

Figure 4 shows a Southern analysis of apple genomic DNA using *MdPI* cDNA as a probe. DNA of Rae Ime (Ri) and Granny Smith (Gs) were digested with EcoRI (E) and HindIII (H).

Figure 5 shows the identification of a transposon insertion in *MdPI* of Rae Ime, Spencer Seedless and Wellington Bloomless.

- Genomic DNA fragments were amplified using primers P3 and P7 from Rae Ime (Ri) and Granny Smith (Gs).
 - (b) Southern blot made from the gel shown in (a) was probed with the cDNA of *MdPI*.

(c) The genomic DNA of *MdPI* from Granny Smith, Rae Ime, Spencer Seedless and Wellington Bloomless was sequenced. The sequence of *MdPI* of Granny Smith was numbered from the ATG start codon. The black boxes are the coding regions and the white box is the 3' non-coding region. A transposon insertion was found in the intron 4 of *MdPI* of Rae Ime and in the intron 6 of Spencer Seedless (Ss) and Wellington Bloomless (Wb) as shown by the arrows.

Figure 6 shows the cDNA and deduced amino acid sequences of MdAP3.

DESCRIPTION OF THE INVENTION

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As broadly outlined above, the applicants have identified a peptide which is involved in fruiting plant reproduction, together with the gene coding therefor. The

specific peptide and gene are from a plant which produces pome fruit, Malus x domestica.

The amino acid sequence of one peptide, *MdPl*, and its encoding nucleotide sequence are given in Figure 2. It will however be appreciated that the invention is not restricted only to the peptide/polynucleotide having the specific amino acid/nucleotide sequence given in Figure 2. Instead, the invention also extends to functionally equivalent variants of the peptide/polynucleotide of Figure 2.

The term "polynucleotide(s)" as used herein means a single or double-stranded polymer of deoxyribonucleotide or ribonucleotide bases and includes DNA and corresponding RNA molecules, including HnRNA and mRNA molecules, both sense and anti-sense strands, and comprehends cDNA, genomic DNA and recombinant DNA, as well as wholly or partially synthesized polynucleotides. An HnRNA molecule contains introns and corresponds to a DNA molecule in a generally one-to-one manner. An mRNA molecule corresponds to an HnRNA and DNA molecule from which the introns have been excised. A polynucleotide may consist of an entire gene, or any portion thereof. Operable anti-sense polynucleotides may comprise a fragment of the corresponding polynucleotide, and the definition of "polynucleotide" therefore includes all such operable anti-sense fragments.

The phrase "functionally equivalent variants" recognises that it is possible to vary the amino acid/nucleotide sequence of a peptide while retaining substantially equivalent functionality. For example, a peptide can be considered a functional equivalent of another peptide for a specific function if the equivalent peptide is immunologically cross-reactive with and has at least substantially the same function as the original peptide. The equivalent can be, for example, a fragment of the peptide, a fusion of the peptide with another peptide or carrier, or a fusion of a fragment which additional amino acids.

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It will of course be understood that a variety of substitutions of amino acids is possible while preserving the structure responsible for activity of the peptide. Conservative substitutions are described in the patent literature, as for example, in United States Patent No 5,264,558 or 5,487,983. It is thus expected, for example, that interchange among non-polar aliphatic neutral amino acids, glycine, alanine,

proline, valine and isoleucine, would be possible. Likewise, substitutions among the polar aliphatic neutral amino acids, serine, threonine, methionine, asparagine and glutamine could be made. Substitutions among the charged acidic amino acids, aspartic acid and glutamic acid, could probably be made, as could substitutions among the charges basic amino acids, lysine and arginine. Substitutions among the aromatic amino acids, including phenylalanine, histidine, tryptophan and tyrosine are also possible. Such substitutions and interchanges are well known to those skilled in the art.

Equally, nucleotide sequences encoding a particular product can vary significantly simply due to the degeneracy of the nucleic acid code.

Variants can have a greater or lesser degree of homology as between the variant amino acid/nucleotide sequence and the original.

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Polynucleotide or polypeptide sequences may be aligned, and percentage of identical nucleotides in a specified region may be determined against another sequence, using computer algorithms that are publicly available. Two exemplary algorithms for aligning and identifying the similarity of polynucleotide sequences are the BLASTN and FASTA algorithms. The similarity of polypeptide sequences may be examined using the BLASTP algorithm. Both the BLASTN and BLASTP software are available on the NCBI anonymous FTP server (ftp://ncbi.nlm.nih.gov) under /blast/executables/. The BLASTN algorithm version 2.0.4 [Feb-24-1998], set to the default parameters described in the documentation of variants according to the present invention. The use of the BLAST family of algorithms, including BLASTN BLASTP, is described at NCBI's website and http://www.ncbi.nlm.nih.gov/BLAST/newblast.html and in the publication of Altschul, Stephen F., et al. (1997), "Gapped BLAST and PSI-BLAST: a new generation 'of protein database search programs", Nucleic Acids Res. 25:3389-34023. The computer algorithm FASTA is available on the Internet at the ftp site ftp://ftp.virginia.edu/pub/fasta/. Version 2.0u4, February 1996, set to the default parameters described in the documentation and distributed with the algorithm, is also preferred for use in the determination of variants according to the present invention. The use of the FASTA algorithm is described in W. R. Pearson and D. J. Lipman, "Improved Tools for Biological Sequence Analysis", Proc.

Natl. Acad. Sci. USA 85:2444-2448 (1988) and W. R. Pearson, "Rapid and Sensitive Sequence Comparison with FASTP and FASTA, "Methods in Enzymology 183:63-98 (1990).

- The following running parameters are preferred for determination of alignments and similarities using BLASTN that contribute to E values (as discussed below) and percentage identity: Unix running command: blastall -p blastn -d embldb -e 10 -G 1 -E 1 -r 2 -v 50 -b 50 -I queryseq -o results; and parameter default values:
 - -p Program Name [String]
- 10 -d Database [String]
 - -e Expectation value (E) [Real]
 - -G Cost to open a gap (zero invokes default behaviour) [Integer]
 - -E Cost to extend a cap (zero invokes default behaviour) [Integer]
 - -r Reward for a nucleotide match (blastn only) [Integer]
- 15 -v Number of one-line descriptions (V) [Integer]
 - -b Number of alignments to show (B) [Integer]
 - -i Ouery File [File In]
 - -o BLAST report Output File [File Out] Optional

For BLASTP the following running parameters are preferred: blastall -p blastp -d

- 20 swissprotdb -e 10 -G 1 -E 1 -v 50 -b 50 -I queryseq -o results
 - -p Program Name [String]
 - -d Database [String]
 - -e Expectation value (E) [Real]
 - -G Cost to open a gap (zero invokes default behaviour) [Integer]
- 25 -E Cost to extend a cap (zero invokes default behaviour) [Integer]
 - -v Number of one-line descriptions (v) [Integer]
 - -b Number of alignments to show (b) [Integer]
 - -i Query File [File In]

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-o BLAST report Output File [File Out] Optional

The "hits" to one or more database sequences by a queried sequence produced by BLASTN, BLASTP, FASTA, or a similar algorithm, align and identify similar portions of sequences. The hits are arranged in order of the degree of similarity and the length of sequence overlap. Hits to a database sequence generally represent an overlap over only a fraction of the sequence length of the queried sequence.

The BLASTN and FASTA algorithms also produce "Expect" or E values for alignments. The E value indicates the number of hits one can "expect" to see over a certain number of contiguous sequences by chance when searching a database of a certain size. The Expect value is used as a significance threshold for determining whether the hit to a database, such as the preferred EMBL database, indicates true similarity. For example, an E value of 0.1 assigned to a hit is interpreted as meaning that in a database of the size of the EMBL database, one might expect to see 0.1 matches over the aligned portion of the sequence with a similar score simply by chance. By this criterion, the aligned and matched portions of the sequences then have a 90% probability of being the same. For sequences having an E value of 0.01 or less over aligned and matched portions, the probability of finding a match by chance in the EMBL database is 1% or less using the BLASTN or FASTA algorithm.

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According to one embodiment, "variant" polynucleotides, with reference to each of the polynucleotides of the present invention, preferably comprise sequences having the same number or fewer nucleic acids than each of the polynucleotides of the present invention and producing an E value of 0.01 or less when compared to the polynucleotide of the present invention. That is, a variant polynucleotide is any sequence that has at least a 99% probability of being the same as the polynucleotide of the present invention, measured as having an E value of 0.01 or less using the BLASTN or FASTA algorithms set at the parameters discussed above.

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Variant polynucleotide sequences will generally hybridize to the recited polynucleotide sequence under stringent conditions. As used herein, "stringent conditions" refers to prewashing in a solution of 6X SSC, 0.2% SDS; hybridizing at 65°C, 6X SSC, 0.2% SDS overnight; followed by two washes of 30 minutes each in 1X SSC, 0.1% SDS at 65°C and two washes of 30 minutes each in 0.2X SSC, 0.1% SDS at 65°C.

It is of course expressly contemplated that homologs to *MdPI* exist in other fruiting plants. Such homologs are also "functionally equivalent variants" of *MdPI* as the phrase is used herein.

DNA sequences from fruiting plants other than *Malus x domestica* which are homologs of *MdPI* may be isolated by high throughput sequencing of cDNA libraries prepared from such plants. Alternatively, oligonucleotide probes based on the sequences for *MdPI* provided in Figure 2 can be synthesized and used to identify positive clones in either cDNA or genomic DNA libraries from other plants by means of hybridization or PCR techniques. Probes should be at least about 10, preferably at least about 15 and most preferably at least about 20 nucleotides in length. Hybridization and PCR techniques suitable for use with such oligonucleotide probes are well known in the art. Positive clones may be analyzed by restriction enzyme digestion, DNA sequencing or the like.

The polynucleotides of the present invention may be generated by synthetic means using techniques well known in the art. Equipment for automated synthesis of oligonucleotides is commercially available from suppliers such as Perkin Elmer/Applied Biosystems Division (Foster City, CA) and may be operated according to the manufacturer's instructions.

The primary importance of identification of the peptide/polynucleotides of the invention is that they enable the reproductive (seed-producing) capacity of fruiting plants to be modulated. This modulation will generally involve a reduction in the functional expression (silencing) of the reproductive peptide.

Any conventional technique for effecting this can be employed. Intervention can occur post-transcriptionally or pre-transcriptionally. Further, intervention can be focused upon the gene itself or on regulatory elements associated with the gene and which have an effect on expression of the encoded peptide. "Regulatory elements" is used here in the widest possible sense and includes other genes which interact with the gene of interest. For example, intervention which targets expression of MdAP3 peptide is contemplated. MdAP3 is functionally related to MdPI such that down-regulation of MdAP3 expression will in turn down-regulate MdPI (see Jack et al (1992) and Goto & Meyerowitz (1994)).

The cDNA and deduced amino acid sequences for MdAP3 are shown in Figure 6.

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Pre-transcription intervention can involve mutation of the gene itself or of its Such mutations can be point mutations, frameshift regulatory elements. mutations, insertion mutations or deletion mutations. These latter mutations include so call "knock-out" mutations in which the gene is entirely ablated.

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Examples of post-transcription interventions include co-suppression or anti-sense strategies, a dominant negative approach, or techniques which involve ribozymes to digest, or otherwise be lethal to, RNA post-transcription of the target gene.

Co-suppression can be effected in a manner similar to that discussed, for example, 10 by Napoli et al (Plant Cell 2:279-290, 1990) and de Carvalho Niebel et al (Plant Cell 7:347-258, 1995). In some cases, it can involve overexpression of the gene of interest through use of a constitutive promoter. It can also involve transformation of a plant with a non-coding region of the gene, such as an intron from the gene or

5'-non-coding leader sequences. 15

> Anti-sense strategies involve expression or transcription of DNA with the expression/transcription product being capable of interfering with translation of mRNA transcribed from the target gene. This will normally be through the expression/transcription product hybridising to and forming a duplex with the target mRNA.

> The expression/transcription product can be a relatively small molecule and still be capable of disrupting mRNA translation. However, the same result is achieved by expressing the target gene in an anti-sense orientation such that the RNA produced by transcription of the anti-sense oriented gene is complementary to all or part of the endogenous target mRNA.

Anti-sense strategies are described generally by Robinson-Benion et al., (1995), Anti-sense techniques, Methods in Enzymol. 254(23):363-375 and Kawasaki et al., (1996), Artific. Organs 20 (8): 836-848.

Dominant negative approaches involve the expression of a modified DNA binding/activating protein which includes a DNA binding domain but not a activator domain. The result is that the protein binds to DNA as intended but fails

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to activate, while at the same time blocking the binding of the DNA binding/activating peptides which normally bind to the same site.

The ribozyme approach to regulation of peptide expression involves inserting appropriate sequences or subsequences (eg. DNA or RNA) in ribozyme constructs (McIntyre CL, Manners JM, *Transgenic Res.* 5(4):257-262, 1996). Ribozymes are synthetic RNA molecules that comprise a hybridizing region complementary to two regions, each of which comprises at least 5 contiguous nucleotides of a mRNA molecule encoded by one of the inventive polynucleotides. Ribozymes possess highly specific endonuclease activity, which autocatalytically cleaves the mRNA.

To give effect to the above strategies, the invention also provides DNA constructs. The constructs include the intended DNA (such as the gene of the invention in anti-sense orientation or a polynucleotide encoding the appropriate DNA binding domain or ribozyme), a promoter sequence and a termination sequence, operably linked to the DNA sequence to be transcribed, which control expression of the gene. The promoter sequence is generally positioned at the 5' end of the DNA sequence to be transcribed, and is employed to initiate transcription of the DNA sequence. Promoter sequences are generally found in the 5' non-coding region of a gene but they may exist in introns (Luehrsen, K.R., Mol. Gen. Genet. 225:81-93, 1991) or in the coding region. When the construct includes an open reading frame in a sense orientation (for co-suppression through over-expression) the promoter sequence also initiates translation of the open reading frame. For DNA constructs comprising either an open reading frame in an anti-sense orientation or a non-coding region, the promoter sequence generally consists only of a transcription initiation site having a RNA polymerase binding site.

A variety of promoter sequences which may be usefully employed in the DNA constructs of the present invention are well known in the art. The promoter sequence, and also the termination sequence, may be endogenous to the target Malus plant host or may be exogenous, provided the promoter is functional in the target host. For example, the promoter and termination sequences may be from other plant species, plant viruses, bacterial plasmids and the like. Preferably, promoter and termination sequences are those endogenously associated with the reproductive genes.

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Factors influencing the choice of promoter include the desired tissue specificity of the construct, and the timing of transcription and translation. For example, constitutive promoters, such as the 35S Cauliflower Mosaic Virus (CaMV 35S) promoter, will affect the activity in all parts of the plant. Use of a tissue specific promoter will result in production of the desired sense or antisense RNA only in With DNA constructs employing inducible promoter the tissue of interest. sequences, the rate of RNA polymerase binding and initiation can be modulated by external stimuli, such as light, heat, anaerobic stress, alteration in nutrient conditions and the like. Temporally regulated promoters can be employed to effect modulation of the rate of RNA polymerase binding and initiation at a specific time during development of a transformed cell. Preferably, the original promoters from the gene in question, or promoters from a specific tissue-targeted gene in the organism to be transformed are used. Other examples of promoters which may be usefully employed in the present invention include, mannopine synthase (mas), octopine synthase (ocs) and those reviewed by Chua et al. (Science, 244:174-181, 1989).

The termination sequence, which is located 3' to the DNA sequence to be transcribed, may come from the same gene as the promoter sequence or may be from a different gene. Many termination sequences known in the art may be usefully employed in the present invention, such as the 3' end of the Agrobacterium tumefaciens nopaline synthase gene. However, preferred termination sequences are those from the original gene or from the target Malus species to be transformed.

The DNA constructs of the present invention may also contain a selection marker that is effective in plant cells, to allow for the detection of transformed cells containing the construct. Such markers, which are well known in the art, typically confer resistance to one or more toxins. One example of such a marker is the NPTII gene whose expression results in resistance to kanamycin or hygromycin, antibiotics which is usually toxic to plant cells at a moderate concentration (Rogers et al., in Methods for Plant Molecular Biology, A Weissbach and H Weissbach eds, Academic Press Inc., San Diego, CA (1988)). Alternatively, the presence of the

desired construct in transformed cells can be determined by means of other techniques well known in the art, such as Southern and Western blots.

Techniques for operatively linking the components of the inventive DNA constructs are well known in the art and include the use of synthetic linkers containing one or more restriction endonuclease sites as described, for example, by Maniatis et al., (Molecular Cloning: A Laboratory Manual, Cold Spring Harbour Laboratories, Cold Spring Harbour, NY, 1989). The DNA construct may be linked to a vector having at least one replication system, for example, E. coli, whereby after each manipulation, the resulting construct can be cloned and sequenced and the correctness of the manipulation determined.

The DNA constructs of the present invention may be used to transform a variety of fruiting plants. In a preferred embodiment, the DNA constructs are employed to transform apple and its related species such as pear.

As discussed above, transformation of a fruiting plant with a DNA construct including an open reading frame coding for a peptide encoded by a DNA sequence of the invention wherein the open reading frame is orientated in a sense direction can, in some cases, lead to a decrease in expression of the peptide by cosuppression. Transformation of the plant with a DNA construct comprising an open reading frame in an anti-sense orientation or a non-coding (untranslated) region of a gene will lead to a decrease in the expression of the peptide in the transformed plant.

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Techniques for stably incorporating DNA constructs into the genome of target fruiting plants are well known in the art and include Agrobacterium tumefaciens mediated introduction, electroporation, protoplast fusion, injection into reproductive organs, injection into immature embryos, high velocity projectile introduction and the like. The choice of technique will depend upon the target plant to be transformed.

Once the cells are transformed, cells having the DNA construct incorporated into their genome may be selected by means of a marker, such as the kanamycin resistance marker discussed above. Transgenic cells may then be cultured in an

appropriate medium to regenerate whole plants, using techniques well known in the art. In the case of protoplasts, the cell wall is allowed to reform under appropriate osmotic conditions. In the case of seeds or embryos, an appropriate germination or callus initation medium is employed. For explants, an appropriate regeneration medium is used.

For a review of regeneration of trees, see Dunstan *et al.*, Somatic embryogenesis in woody plants. In: Thorpe, T.A. ed. 1995: *in vitro* embryogenesis of plants. Vol 20 in Current Plant Science and Biotechnology in Agriculture, Chapter 12, pp. 471-540.

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The resulting transformed fruiting plants may be reproduced sexually or asexually, using methods well known in the art, to give successive generations of transgenic plants.

The nucleotide sequence information provided herein will also be useful in

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programs for identifying nucleic acid variants from fruiting plants and for preselecting plants with mutations in *MdPl*, *MdAP3* or their equivalents which renders those plants useful in an accelerated breeding program to produce seedless fruit. More particularly, the nucleotide sequence information provided herein may be used to design probes and primers for probing or amplification of *MdPl*, *MdAP3* or variants thereof. An oligonucleotide for use in probing or PCR may be about 30 or fewer nucleotides in length. Generally, specific primers are upwards of 14 nucleotides in length. For optimum specificity and cost effectiveness, primers or 16-24 nucleotides in length are preferred. Those skilled in the art are well versed in the design of primers for use in processes such as PCR.

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If required, probing can be done with entire restriction fragments of the gene disclosed herein. Naturally, sequences based upon Figure 2, or Figure 6 or the complements thereof can be used.

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Such probes and primers also form aspects of the present invention.

Probing may employ the standard Southern blotting technique. For instance, DNA may be extracted from cells and digested with different restriction enzymes.

35 Restriction fragments may then be separated by electrophoresis on an agarose gel,

before denaturation and transfer to a nitrocellulose filter. Labelled probes may be hybridised to the DNA fragments on the filter and binding determined. DNA for probing may be prepared from RNA preparations from cells. Probing may optionally be done by means of so-called "nucleic acid chips" (see Marshall and Hodgson (1998)).

The invention will now be illustrated with reference to the following non-limiting experiments.

10 EXPERIMENTAL

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Methods and Materials

Cloning MdPI using PCR approaches

Total RNA was isolated from 'Granny Smith' apple flowers using the method described by Chang et al (1993). Poly(A) mRNA was purified from the total RNA using the mRNA Purification Kit (Pharmacia, Sweden). cDNA was synthesized from the mRNA using the ZAP cDNA Synthesis Kit (Stratagene, CA, USA). DNA fragments were amplified from templates of cDNA using two degenerative PCR primers P1 CGGAATTCATGGGNMGNGGNAARRT-3' and P2

primers P1 CGGAATTCATGGGNMGNGGNAARRT-3' and P2 CGCTCGAGGATCCGGYTGNATNGGYTGNAC-3' (N=ATGC, M=AC, R=AG, Y=CT). The primers were designed according the conserved amino acid sequences MGRGKI in the MADS-box domain and VQPM/IQP in the C-terminal region (Fig. 2) in an alignment of PI, GLOBSA, FBP3, SLM2 and pMADS2. The underlined Eco RI and Bam HI sites were used for cloning the PCR products. The PCR amplification conditions were as follows: initial denaturation at 94°C for 4 min; then 35 cycles of 94°C for 1 min, 60°C for 1 min, and 72°C for 3 min, and with a final extension of 5 min at 72°C. Several bands were detected from the PCR on agarose gels and DNA in a band of the expected size (630 bp) was cloned into Bluescript SK (Stratagene,

CA, USA) following Eco RI and Bam HI digestion. After the sequences of cloned fragments were determined, two nested PCR primers, P3 and P4 (Fig. 2) were designed using the sequences within the K-box and were used to amplify the 3' region of MdPI cDNA together with a 3' RACE primer GAGAGAGAACTAGTCTCGAG-3'. The PCR conditions were the same as above except for the anneal temperature

reduced to 50°C. The amplified fragments were cloned into pGEM-T EASY Vector (Promega).

Genomic fragments of MdPI were amplified using primers P5 and P6, P3 and P7 (Fig. 2). PCR conditions were: initial denaturation at 94°C for 2 min; then 10 cycles of 94°C for 15 sec, 58°C for 30 sec; and 20 cycles of 94°C for 15 sec, 58°C for 30 sec and 68°C for 5 min plus cycle elongation of 20 sec for each cycle; and with a final extension of 5 min at 86°C. The amplified fragments were cloned into pGEM-T EASY Vector. Expand High Fidelity PCR System (Boehringer Mannheim) was used for all PCR experiments.

DNA sequence determination

Nucleotide sequences of MdPI clones were determined using the automatic sequencer ABI PRISM model 377(CA, USA) with universal forward and reverse primers. To obtain complete sequences, gene specific primers were designed and ordered from BRL Life Technologies.

Northern and Southern analysis using MdPI on apple tissues

Total RNA was isolated as described by Chang et al (1993) from 'Granny Smith' and Rae Ime apple tissues. Northern blots were prepared as described by Dong et al (1997). The northern blot contained RNA isolated from expanding leaves, unopened flowers, and fruit at 2 days and 1, 4 and 8 weeks following hand-pollination. At 4 weeks after pollination, apple fruit is large enough to allow for easy separation into the three main tissue types namely; core, cortex and skin.

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DNA was isolated from leaf tissue of Granny Smith and Rae Ime using the method of Rogers and Bendich (1988). Southern blots were prepared by digesting apple DNA (approximately 20 µg per lane) with EcoRI or HindIII, separating DNA fragments on 0.7% agarose gel and transferring them to Hybond-N+ membrane.

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Northern and Southern blots were probed with 32P-dCTP labelled PI cDNA clone lacking the MADS-box sequence to significantly reduce cross hybridization32P-dCTP labelled MADS-box DNA fragments. The blots were hybridized in 0.5M NaPO4 buffer (pH 7.2) with 1 mM EDTA and 7% SDS at 65°C and washed using 0.4x SSC

and 0.2% SDS at 65°C. Hybridisation signals were detected using a Storm 840 PhosphorImager (Molecular Dynamics, Sunnyvale, California, USA).

Results/Discussion

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Flowers of the majority of apple taxa bear 5 sepals, 5 petals, 9-20 stamens (Fig. 1a) and an inferior ovary. These flowers develop into a pome fruit that consists of fleshy cortex tissue derived from the fused bases of sepals, petals and stamens, and the core tissue derived from fertilised ovary containing 5 carpels and up to 10 seeds (Pratt, 1988) (Fig. 1b). In contrast, flowers of Rae Ime show no petal or stamens but increased numbers of styles (Fig. 1c). These flowers develop into seedless fruit without the need for pollination. These seedless fruit have two whorls of carpels, five carpels in the lower whorl and 9 to 10 in the upper whorl (Fig. 1d). The fruit also has duplicated whorls of calyxes (Fig. 1e) that are the remains of sepals, compared to one calyx whorl in a normal apple (Fig. 1f). The mature seedless fruit are close to normal apple fruit size, but the fruit cores are relatively smaller (Fig. 1g).

Several apple varieties, such as Spencer Seedless and Wellington Bloomless (Tobutt, 1994), have been described with a very similar flower and fruit structure to that of Rae Ime. Anatomy studies of the vascular connections show that the upper whorl of carpels has been transformed from the stamens and the second whorl of sepals from petals (Brase, 1937). In the Arabidopsis pi and ap3 mutants, flowers have no petals or stamens but have double the number of sepals and carpels (Goto and Meyerowitz, 1994; Jack et al., 1992).

A difference between Rae Ime apple and pi Arabidopsis is that the former produces parthenocarpic fruit but the latter does not. Up to 6 apple varieties have been recorded to produce apetalous flowers and parthenocarpic fruit in different countries. Many of these records can be traced back to several centuries ago (Brase, 1937; Tobutt, 1994). This indicates some of the apple mutants may have occurred independently.

Genetic analysis has been performed using two apetalous/parthenocarpic varieties, Spencer Seedless and Wellington Bloomless. Crossing pollen from the

cultivar Wijcik with normal flowers to Wellington Bloomless generates hybrids that all produce normal flowers. Crossing the pollen from these hybrids to Spencer Seedless generates plants of which half produce normal flowers and half produce apetalous flowers and parthenocarpic fruit (Tobutt, 1994). This result indicates that a single recessive gene controls apetalous flower development and subsequently parthenocarpic fruit formation. This result also indicates that mutations in Spencer Seedless and Wellington Bloomless are different alleles at the same locus. Independently isolated mutant alleles at the same locus are good evidences for a single gene being involved in the development of apetalous flower and parthenocarpic fruit in these apple mutants.

DNA fragments of 630bp have been amplified from apple flower cDNA using degenerative PCR primers against conserved sequences in the MADS-box and in the C-terminal region of PI and its homologues. After these DNA fragments were cloned, 6 random clones were sequenced and found to contain the same sequences. The cloned cDNA sequences started from the first presumed ATG start coden, contained MADS-box, K-box and most of the C-terminal region and had high homology to PI. The C-terminal and the 3' un-translated regions were further amplified using two nested PCR primers within the K-box and a 3' RACE primer. Six clones containing the 3' fragments were sequenced and found to contain the same sequences overlapping with those in the 5' clone. Sequences from the 5' and 3' clone were assembled together and shown in Fig. 2. These sequences show highest homology to PI and its homologues (GLOBOSA, FBP3, SLM2 and pMADS2) in Blast searches carried out in GeneBank. The putative apple PI homologue was named as MdPI having a deduced amino acid sequence identity of 64% to that of Arabidopsis PI protein.

MdPI is found to be highly expressed in petals and stamens as determined through northern analysis. Expression in other apple tissues, including sepals and ovaries, is either not detected or found to be very low (Fig. 3). This expression pattern is essentially the same as that shown for Arabidopsis PI gene (Goto and Meyerowitz, 1993). Genomic sequences of MdPI were amplified using the PCR primers P5 within the MADS-box and P6 within the 3' non-translated region. Two clones containing the MdPI genomic DNA were sequenced and found to contain the same sequences having six easily identifiable introns. The relative positions of intron 2

to intron 6, are highly conserved compared to the positions of 5 introns in PI gene (Fig. 2). We conclude that MdPI is the PI homolog based on these results having highest sequence identity and conserved intron positions and mRNA expression patterns.

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In an experiment to examine whether there is a mutation in *MdPI* of Rae Ime, the expression level of *MdPI* in flower buds was determined. Expression of *MdPI* in the apetalous Rae Ime flower buds is not detected, but is readily detected in normal flower buds of the Granny Smith variety (Fig. 3). In *Arabidopsis pi* mutants, *PI* expression is reduced or abolished in flower buds (Goto and Meyerowitz, 1994).

A second experiment compared RFLP patterns for Rae Ime with normal apple cultivars using the *MdPl* cDNA as a probe. Southern hybridisation shows different RFLP patterns between Rae Ime and Granny Smith with both *Eco*RI and *HindIII* digestion (Fig. 4) although Granny Smith RFLP pattern is conserved in another apple variety Royal Gala (data not shown). Both the expression and RFLP data indicate that the *MdPl* gene in Rae Ime has been mutated. As both enzyme digestions reveal RFLP differences, the mutation is likely to be a gross change in gene structure rather than a point mutation

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Genomic DNA fragments were cloned from Granny Smith and Rae Ime using two primers P3 and P7 designed with *MdPI* cDNA sequence. The Rae Ime fragments were 11 kb while the Granny Smith fragments were 2 kb (Fig. 5a). These fragments show a hybridisation signal to the *MdPI* cDNA probe (Fig. 5b). Clones containing these fragments were partially sequenced from two ends. The Rae Ime fragments have the same sequence to the Granny Smith fragments at two ends, but with an insertion in the intron 4 of *MdPI* gene in Rae Ime (Fig. 5b). The insertion sequences were found to be an LTR retrotransposon. This result confirmed that there is a mutation in the *MdPI* gene in Rae Ime.

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By way of confirmation that it is the mutation of the *MdPI* gene which is responsible for the parthenocarpic phenotype, the *MdPI* gene from two further parthenocarpic apple varieties, Spencer Seedless and Wellington Bloomless, was sequenced (data not shown). This revealed an approximately 9 kb insertion in each gene. Thus, in the three parthenocarpic apple varieties examined, there are

two different insertion sites in the *MdPI* gene both of which lead to the parthenocarpic phenotype. Spencer Seedless and Wellington Bloomless have the same insertion site, which is different from that in Rae Ime (Fig. 5c). These confirmatory results demonstrate that independent mutations in *MdPI* generate the same apetalous/parthenocarpic phenotype.

The difference in fruit development between Rae Ime apple and pi Arabidopsis may be explained in two different ways. Firstly, MdPI may have different function compared to PI in influencing ovary and fruit development. Sufficient functional differences have been shown for homologs of floral homeotic genes in different plant species (Causier et al., 1999). Secondly, apple fruit develops from both ovary and the fused bases of sepals, petals and stamens (Pratt, 1988). Apple differs from tomato and Arabidopsis, two model systems often used in studies of fruit development, where the fruit or silique develops from ovary tissue only (Weigel and Mererowitz, 1994; Gillaspy et al., 1993). The differences in fruit structure may cause different fruit development after a mutation in a floral homeotic gene.

INDUSTRIAL APPLICATION

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20 In its primary aspect, the invention has application in modulating, and in particular reducing or eliminating seed-bearing capacity in fruiting plants. Such plants have utility in horticulture.

It will also be possible to employ the polynucleotides of the invention in breeding programmes to monitor the progress made towards breeding a stable seedless fruiting plant.

The availability of reproductively null or sterile trees has the additional advantage that it will be possible to introduce further exogenous genetic material into those trees without the risk that the material will be passed on to other trees.

Those persons skilled in the art will appreciate that the specific description provided is exemplary only, and that modifications and variations may be made without departing from the scope of the invention.

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CLAIMS:

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 A fruiting plant which has been genetically modified such that it does not functionally express:

- (i) a peptide having the *MdPI* amino acid sequence of SEQ ID NO: 2 or a functionally equivalent variant thereof; and/or
- (ii) a peptide having the MdAP3 amino acid sequence of SEQ ID NO: 4 or a functionally equivalent variant thereof,

which plant produces seedless or sterile fruit.

- 2. A fruiting plant which contains a polynucleotide encoding a peptide having the *MdPl* amino acid sequence of SEQ ID NO: 2 or a functionally equivalent variant thereof and in which the functional expression of said peptide within said plant has been disrupted such that the plant produces seedless or sterile fruit.
 - 3. A fruiting plant according to claim 1 or claim 2 which produces a pome fruit.
- 15 4. A fruiting plant which contains:
 - (a) a polynucleotide encoding a peptide having the *MdPI* amino acid sequence of SEQ ID NO: 2 or a functionally equivalent variant thereof; and
- (b) a polynucleotide encoding a peptide having the MdAP3 amino acid sequence of SEQ ID NO: 4 or a functionally equivalent variant thereof,

and in which the functional expression of said peptide encoded by polynucleotide (a) within said plant has been disrupted such that the plant produces seedless or sterile fruit.

- 25 5. A plant as claimed in claim 4 wherein functional expression of said peptide encoded by polynucleotide (a) is disrupted directly.
 - 6. A plant as claimed in claim 4 wherein functional expression of said peptide encoded by polynculeotide (a) is disrupted indirectly.

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7. A plant as claimed in claim 6 wherein said indirect disruption is effected through disrupting functional expression of the peptide encoded by said polynucleotide (b).

- 8. A plant as claimed in any one of claims 4 to 7 wherein said plant is one which produces pome fruit.
 - 9. A plant as claimed in claim 8 wherein said polynucleotide (a) has the coding sequence of SEQ ID NO: 1.
 - 10. A plant as claimed in claim 8 wherein said polynucleotide (a) has the nucleotide sequence of SEQ ID NO: 1.
- 10 11. A plant as claimed in claim 8, claim 9 or claim 10 in which said polynucleotide (b) has the coding sequence of SEQ ID NO: 3.
 - 12. A plant as claimed in claim 8, claim 9 or claim 10 wherein said polynucleotide (b) has the nucleotide sequence of SEQ ID NO: 3.
- 13. A polynucleotide which encodes a peptide having the *MdPI* amino acid sequence of SEQ ID NO: 2 or a functionally equivalent variant thereof.
 - 14. A polynucleotide as claimed in claim 13 which comprises the coding sequence of SEQ ID NO: 1.
 - 15. A polynucleotide as claimed in claim 13 which comprises the nucleotide sequence of SEQ ID NO: 1.
- 20 16. A polynucleotide which encodes a peptide having the *MdAP3* amino acid sequence of SEQ ID NO: 4 or a functionally equivalent variant thereof.
 - 17. A polynucleotide as claimed in claim 16 which comprises the coding sequence of SEQ ID NO: 3.
- 18. A polynucleotide as claimed in claim 16 which comprises the nucleotide sequence of SEQ ID NO: 3.
 - 19. A DNA construct which includes a polynucleotide as claimed in any one of claims 13 to 18.

- 20. A DNA construct comprising, in the 5'-3' direction:
 - (a) a promoter sequence;
 - (b) an open reading frame polynucleotide as defined in any one of claims 13 to 18; and
- 5 (c) a termination sequence.

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- 21. A DNA construct as claimed in claim 20 wherein the open reading frame polynucleotide is in a sense orientation.
- 22. A DNA construct as claimed in claim 20 in which the open reading frame polynucleotide is in an anti-sense orientation.
- 10 23. A DNA construct comprising, in the 5'-3' direction:
 - (a) a promoter sequence;
 - (b) a non-coding region of a gene coding for the peptide having the *MdPI* amino acid sequence of SEQ ID NO: 2 or a functionally equivalent variant thereof; and
- 15 (c) a termination sequence.
 - 24. A DNA construct comprising, in the 5'-3' direction:
 - (a) a promoter sequence;
 - (b) a non-coding region of a gene coding for the peptide having the MdAP3 amino acid sequence of SEQ ID NO: 4 or a functionally equivalent variant thereof; and
 - (c) a termination sequence.
 - 25. A DNA construct as claimed in claim 23 or claim 24 in which the non-coding region is in a sense orientation.
- 26. A DNA construct as claimed in claim 23 or claim 24 in which the non-coding region is in an anti-sense orientation.

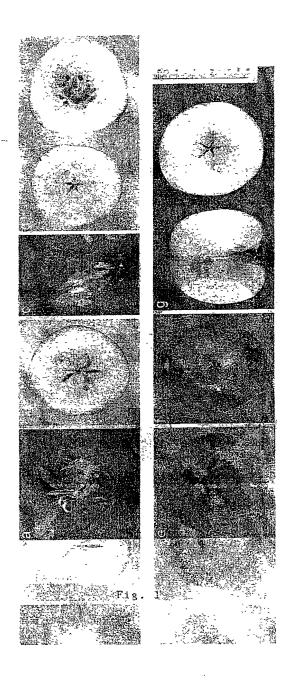
- 27. A DNA construct comprising, in the 5'-3' direction:
 - (a) a promoter sequence;

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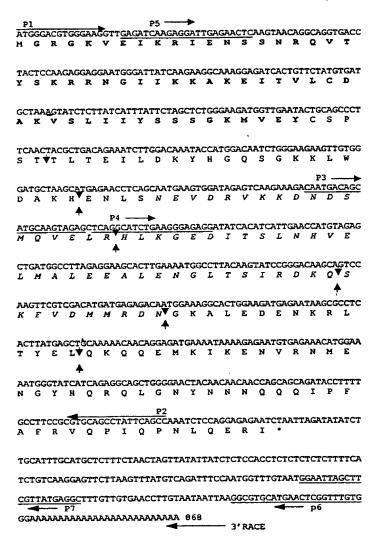
- (b) a polynucleotide comprising a nucleotide sequence complementary to at least part of a sequence coding for the peptide having the *MdPI* amino acid sequence of SEQ ID NO: 2 or a functionally equivalent variant thereof; and
- (c) a termination sequence.
- 28. A DNA construct comprising, in the 5'-3' direction:
 - (a) a promoter sequence;
- 10 (b) a polynucleotide comprising a nucleotide sequence complementary to at least part of a sequence coding for the peptide having the MdAP3 amino acid sequence of SEQ ID NO: 4 or a functionally equivalent variant thereof; and
 - (c) a termination sequence.
- 15 29. A transgenic cell of a fruiting plant which includes a DNA construct as claimed in any one of claims 19 to 28.
 - 30. A transgenic cell as claimed in claim 29 in which said fruiting plant is one which produces a pome fruit.
 - 31. A fruiting plant containing a transgenic cell as claimed in claim 29.
- 20 32. A fruiting plant containing a transgenic cell as claimed in claim 30.
 - A seedless or sterile fruit which is produced by a fruiting plant as claimed in any one of claims 1, 2, 4-7 and 31.
 - 34. A seedless or sterile pome fruit which is produced by a fruiting plant as claimed in any one of claims 3, 8 to 12 and 32.

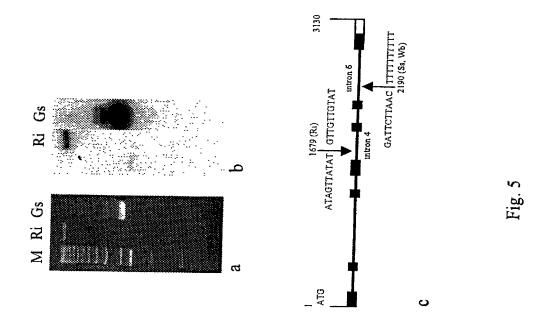
Abstract

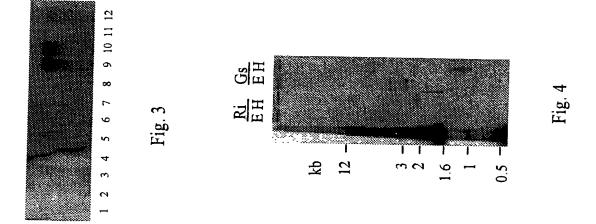
The invention provides fruiting plants that produce seedless or sterile fruit. The production of seedless or sterile fruit is the result of genetic modification which prevents or disrupts functional expression of the MdPI peptide of SEQ ID NO: 2 or a variant thereof, or of the MdAP3 peptide of SEQ ID NO: 4 or a variant thereof, or both.



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MARGKIEIKLIENQTNRQVT TACTCCAAGAGAAATGGGATCTTCAAGAAGGCTCAGGAGCTCACCGTTCTCTGTGAT Y S K R R N G I F K K A Q E L T V L C D GCCAAGGTCTCCCTCATTATGCTCTCCAACACTAATAAAATGCACGAGTATATCAGCCCT A K V S L I M L S N T N K M H E Y I S P TTTTKSMYDDYQKTMGIDLW ${\tt AGGACACGAGGAGTCGATGAAAGACACCTTGTGGAAGTTGAAAGAGATCAACAATAAG}$ RTHEESMKDTLWKLKEINNK CTGAGGAGAGATCAGGCAGAGGTTGGGCCATGATCTAAATGGCCTGAGCTTTGACGAG LRREIRQRLGHDLNGLSFDE CTGGCTTCTCTTGACGATGAGATGCAGTCTTCCTTGGATGCCATACGTCAAAGGAAGTAC LASLDDEMQSSLDAIRQRKY CATGTGATCAAAACTCAGACGGAGACCACCAAGAAGAAGGTTAAGAACTTGGAGCAAAGA H V I K T Q T E T T K K K V K N L E Q R AGAGGAAACATGCTGCATGGCTATTTTGACCAGGAAGCAGCCGGCGAGGATCCACAGTAT RGNMLHGYFDQEAAGEDPQY GGTTATGAGGACAATGAGGGAGACTACGAATCTGCACTTGCATTGTCAAATGGGGCGAAT G Y E D N E G D Y E S A L A L S N G A N NLYTFHLHHPNLHHGGSSLG TCCTCCATTACTCATCTGCACGATCTCCGCCTTGCTTGATCGTGATCTGAGATATGATTA SSITHLHDLRLA* ATCATCACTAAGTTATATATAAGGTCACTTATAACTGCTTTTGCTCTAAAGTGTTTGCT TGGTGACTATCTTTAGGCAAGGAGTTAGACTTGGACTACCTCTGAAAACAGATGCATAAA АТААААААААААААААААА 982

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